MULTIPLE ALEURONE AND OPAQUE-2 MUTANT EFFECTS ON MICRONUTRIENT CONCENTRATION IN MAIZE

A Thesis

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ABSTRACT

Many resource-poor people who subsist mainly on a maize-based diet suffer from micronutrient deficiency, in particular iron deficiency and niacin deficiency. In response, biofortification has been proposed as a sustainable strategy for micronutrient malnutrition intervention. Motivated by this, we studied the effects of the multiple aleurone layer (*Mal*) allele on mineral and total niacin concentrations of maize kernels. We also studied the synergistic effects of *Mal* with the opaque-2 (*o2*) allele on mineral and total niacin concentrations.

For nutritionally important mineral elements, we found no significant effects due to the presence of *Mal* in BC1S1 kernels from the B8 background. Analyzed across Mo17 and W64A inbred backgrounds using S2 kernels, copper concentration was significantly lower in multiple aleurone versus single aleurone phenotypes. Total niacin assay tested across Mo17 and W64A revealed that multiple aleurone layer kernels had significantly higher total niacin concentration (11.3%).

Tested across Mo17o2 and W64Ao2 (in opaque-2 kernels only), magnesium concentration was higher in multiple aleurone versus single aleurone layer phenotype kernels. Potassium concentration was significantly higher in multiple aleurone versus single aleurone layer kernels only in W64Ao2.

Within a given aleurone layer phenotypic class and a given inbred background, *o2* affected concentrations of various mineral nutrients. There was a numeric trend suggesting interaction between *Mal* and *o2* for iron and sulfur concentrations in Mo17o2 and Mo17O2. There was also a numeric trend suggesting interaction between *Mal* and *o2* for iron and potassium concentrations in W64Ao2 and W64AO2. There was significant interaction for manganese concentration between *Mal* and *o2*

in W64Ao2 and W64AO2, with the *mal O2* genotype having higher concentration than *Mal O2*, which in turn was higher than *Mal o2*. As these differences were non-significant, mostly occured in only one inbred background or contradicting in two backgrounds; or the double mutant showed a significant mineral decrease, they do not provide strong support for breeding for both *Mal* and *o2* mutations to improve maize mineral concentrations.

Total niacin concentration was not significantly affected by *Mal* when tested across Mo17o2 and W64Ao2. The *o2* allele increased the niacin concentration in Mo17o2 and W64Ao2 when compared to their non-*o2* counterparts within a given aleurone layer phenotypic class. There was a numerical trend suggesting an interaction between *Mal* and *o2* in both Mo17 and W64A backgrounds, with the double mutant having the highest niacin concentration. Although the double mutant was not significantly higher in total niacin concentration than the *o2* single mutant, given the heterozygosity remaining in these S2 progenies, the numerical superiority of the double mutant suggests that breeding for both *Mal* and *o2* might have potential to increase total niacin concentration in maize kernels.

Our results are limited by the number of inbred backgrounds we were able to work with, the number of ears from each background we were able to assay, and the fact that our progenies had residual background genetic variation (i.e., they were not truly isogenic lines). The evidence for *Mal* effects and *Mal* interaction with *o2* that affects mineral and niacin concentrations is sufficient to justify further research that minimizes these limitations, in order to more fully understand the potential of these two mutations to enhance maize nutritional value.

BIOGRAPHICAL SKETCH

Yunting Dai, known as Ting with her friends and in the department of Plant Breeding and Genetics, was born in Shanghai, China. She graduated from the Second Secondary School Attached to East China Normal University, Shanghai, China in 2001. She received her Bachelor of Agronomy degree from Shanghai Jiaotong University in 2005, majored in Plant Science and Technology. When she was a college student, she was also highly involved in Nongovernmental Organization (NGO) work promoting youth health education domestically and internationally. She participated in the Miracle Corners of the World (MCW) project in Tanzania in 2003 and was an Oxfam International Youth Parliament (OIYP) partner from 2004 to 2006. Her passion and broad interests lead her to doing biofortification research at Cornell.

This master's thesis is dedicated to my parents, family and friends.

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CHAPTER 1

INTRODUCTION

1.1 Nutrition and Agriculture

"Nutrition is a key element to any strategy to reduce the global burden of disease. Hunger, malnutrition, obesity and unsafe food all cause diseases, and better nutrition will translate into large improvements in health among all of us, irrespective of our health and home country," says Dr. Bro Harlem Brundtland (Former Director General, World Health Organization (WHO), United Nations) [1].

The cruel reality is that the quantity of food per capita has been declining since two decades ago while just in this year world hunger is projected to reach a new high with 1030 million people suffering every day (FAO website[1]). The 2008 Global Hunger Index[2](GHI) shows some improvement from the 1990 GHI, but the problem remains serious especially for certain regions—mainly South-East Asia, Sub-Saharan-Africa and Latin America and the Caribbean—and "the world is making slow progress in reducing the food insecurity" [3].

International agriculture researchers believe that agriculture, the primary source of nutrients for human life, has the potential to reduce malnutrition worldwide similar to the past Green Revolution experience [33].

[1] `http://www.fao.org/news/story/en/item/20568/icode/`, July 2009.
[2] GHI is a multidimensional approach to measure hunger and malnutrition.

1.1.1 Malnutrition: The Hidden Hunger

Dorlands Medical Dictionary[3] defines malnutrition as "poor nourishment resulting from an inadequate or improper diet or from some defect in metabolism that prevents the body from using its food properly."

Malnutrition continues to be a severe health problem in developing countries [18]. From 2000 to 2003, 53% of deaths worldwide among children under 5 were attributed to malnutrition (cf. Figure 1.1). Both constituents of malnutrition, i.e. protein-energy malnutrition[4] and micronutrient malnutrition[5], have direct effects on childrens' development and on people of all ages.

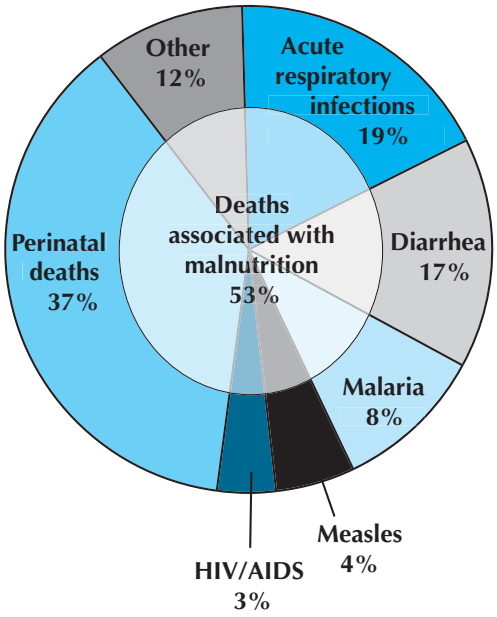


Figure 1.1: **Causes of death among children under 5 years of age, 2000–2003, worldwide [18].**

[3] `http://www.mercksource.com/pp/us/cns/cns_hl_dorlands_split.jsp?pg=/ppdocs/us/common/dorlands/dorland/misc/dmd-a-b-000.htm`, July, 2009.

[4] Protein-energy malnutrition: measurements fall below 2 standard deviations under the normal weight for age (underweight), height for age (stunting) and weight for height (wasting) [18].

[5] Micronutrient malnutrition: Deficiency in minerals and vitamins.

Malnutrition has become a socio-economic problem, requiring huge public cost and causing loss of human capita. Severe cases of malnutrition in a particular country or region are often related with poverty in the given area [4].

Prevalence of Micronutrient Deficiency

Starting from the early 1990s, the phenomenon of "Hidden Hunger" drew more attention owing to the widespread nature of this particular "plague"—billions of people are affected by micronutrient deficiency.

At this time, more than 50% of the world's population is affected by micronutrient deficiency, mainly women, infants and children with few resources [36]. Lack of certain minerals or vitamins may have irreversible consequences in the human body, such as impairment of the immune system (zinc), obstruction of mental and cognitive development (iodine and iron), as well as childhood blindness (vitamin A), to name a few.

Worldwide, nearly two billion people (35.2% of the world's population) do not have sufficient iodine intake [22], two billion suffer from zinc deficiency, and 250 million people are affected by vitamin A deficiency (VAD). Dauntingly, iron deficiency alone is increasing extremely fast among poor women in developing countries according to the 2002 WHO World Health Report [1].

In a more recent publication by WHO, Global Database on Anaemia [12], it is estimated that 1.62 billion people were affected by iron deficiency anaemia (IDA). The highest proportion of individuals affected was found in Africa: 47.5 to 67.6% of the general population, in particular preschool-age children as well as pregnant and non-pregnant women. In in this demographic group (young children and women),

315 million were affected in South-East Asia. Of the surveyed countries, 80% are classified as having a moderate or severe public health problem regarding anaemia.

Many other micronutrient deficiencies exist, mostly in developing countries, but their prevalence is more difficult to assess [4]. Examples include deficiency of folate, vitamin D, niacin, and calcium.

For the "big three" players in micronutrient malnutrition (VAD, IDD, and IDA), the common cause is poor quality of diet [18]. This is especially true for the poor in the developing world, since people with poor resources typically do not have access to sufficiently diverse food (fish, poultry, meat, eggs, milk, different vegetables and fruits, etc.) but rather have to rely on the staple food they have (rice, wheat, maize, sorghum, cassava, etc.), which often cannot provide sufficient micronutrients.

Traditional Micronutrient Deficiency Intervention

Current methods promoted by human nutritionists to cope with world-wide micronutrient malnutrition include: food fortification, dietary supplementation, and dietary diversification [13].

Even in some resource-scarce countries and areas, food fortification can be effective. Vitamin A fortification of sugar in Latin America is an example of an effective long-running fortification program [4]. The universal salt iodization is another one of the more successful examples. In the developing world alone, salt iodization is protecting 82 million newborns every year from cognitive impairment [2].

Despite all its advantages, food fortification does not solve the problems of the

world's poorest populations for multiple reasons. In particular, fortified foods are mostly supplied by the private sector. Because of low local availability and low purchasing power, fortified food is often inaccessible to those who need it the most (see the WHO 2006 Guideline on Micronutrient Fortification [4]).

Dietary supplementation is normally provided through the existing health services and taken orally. Besides the disadvantage of the potentially toxic intake of high levels of certain minerals or vitamins, supplementation needs to be sustained at a steady level of funding year after year [9].

1.1.2 Using Agriculture Against Micronutrient Malnutrition

The advancements of biotechnology promise great improvements of the dysfunctional food system, which would feed the poorest people. Many of the practices reviewed in [37] are conducive to providing more nutrients.

Biofortification, using micronutrient-dense crops as micronutrient vectors, has several inviting advantages [21]:

- It targets low-income households well.
- The germplasm can be shared internationally (cf. high β-carotene South American sweet potato utilized to improve the African materials [16]).
- The biofortified crop system is highly sustainable.
- It can reach remote rural areas.
- It has been reviewed as cost-effective [16].

The HarvestPlus program[6], for instance, is a major research effort designed to reduce the prevalence of micronutrient deficiencies. It continues to support research aimed at increasing the concentrations of iron, zinc, and provitamin A in crops.

1.2 Maize-Based Diet in Developing Countries

In contrast to industrialized nations, maize (*Zea mays* L.) remains a major staple in developing nations. For example, the human maize consumption (Calories/capita/day) in Zambia, Honduras, or Nepal is several times higher than in the United States (cf. Figure 1.2; Data obtained from FAO Food Balance Sheet 2003[7]).

It has also been found that human maize consumption is inversely related with national gross domestic product (GDP) (cf. Figure 1.3; data obtained from FAO Food Balance Sheet 2003[7] and World Facts and Figures[8].). Many of these countries with high intake of maize as a staple are facing the micronutrient malnutrition threat, particularly in South-East Asia, Latin America, and Africa. In these countries, poverty does not allow citizens to improve their quality of diet.

[6] `http://www.harvestplus.org`, July, 2009.
[7] `http://faostat.fao.org/site/368/default.aspx#ancor`, July, 2009.
[8] `http://www.worldfactsandfigures.com/gdp_country_desc.php`, July, 2009.

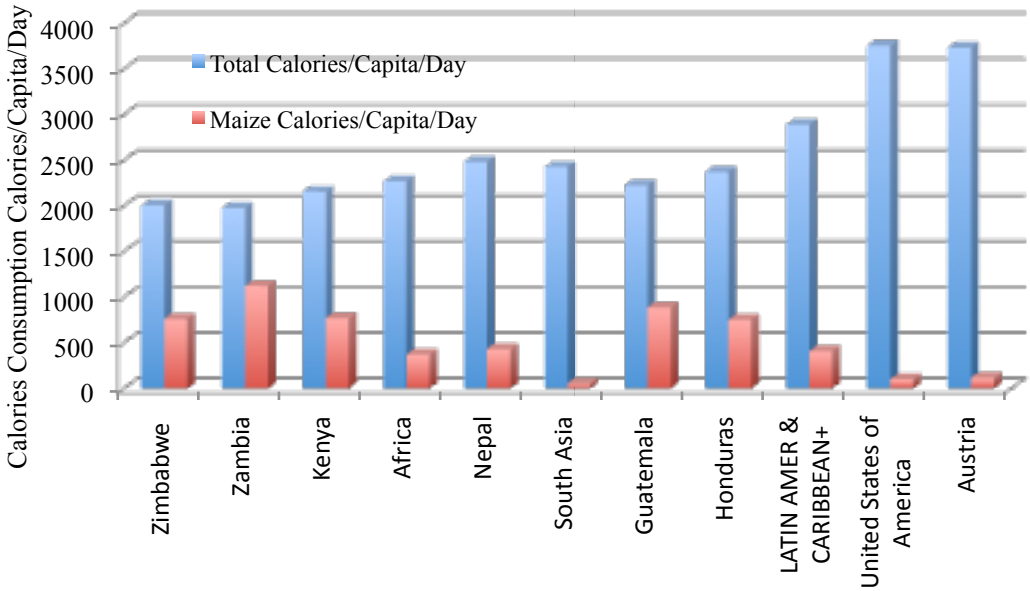


Figure 1.2: **Human maize dietary intake compared to total daily dietary intake from selected countries and regions.**

Figure 1.3: **Statistics on gross domestic product per capita and percentage of maize in the total diet distribution for selected countries.**

To make matters worse, maize kernels are known to have low bioavailable levels of calcium, iron, and zinc [34]. Besides IDA, pellagra[9] prevails among those whose dietary staple is maize, because maize is not rich in niacin and the small amounts of niacin are often in bound form, thus not highly bioavailable [30]. Pellagra is encountered mainly in Africa, India, and parts of China [30].

Improving the nutritional quality of maize may decrease the prevalence of micronutrient deficiency among people with poor resources, subsisting on a maize-based diet (especially women and preschool-age children).

1.3 The State of Micronutrient Improvement Research of Maize

To this date, many efforts have been made to improve the micronutrient content of maize. The International Maize and Wheat Improvement Center (CIMMYT) has focused on identifying maize germplasm with the potential to increase zinc and iron concentrations [5]. Low-phytic-acid (*lpa*) was mutant maize studied with the goal of increasing the bioavailability of several different mineral nutrients (e.g., phytic-acid in the diet lowers the absorption of iron) [24]. This year, a transgenic multi-vitamin maize variety with 169-fold the β-carotene content, six-fold the ascorbate content, and double-fold the folate content of regular maize was reported [19].

[9] Pellagra "rough skin" or niacin deficiency has the following symptoms: weakness, anorexia, indigestion followed by dermatitis, diarrhea and dementia.

1.3.1 The Aleurone Layer

Aleurone is a highly specialized layer of endosperm tissue, which is rich in proteins and lipids [25]. Normally, the aleurone layer in maize is one cell layer thick, covering most of the perimeter of the endosperm.

Several studies investigated the aleurone-layer morphology from a molecular perspective. Scientists at Pioneer Hi-Bred International have confirmed using *Mu* tagging that the *sal1* gene determines the number of aleurone layers in maize endosperm [29]. It encodes a class E vacuolar sorting protein.

Crinkly 4 (*cr4*) [6] and defective kernel 1 (*dek1*) [15] were found to be essential for the aleurone layer formation. However, micronutrient studies were not performed at a molecular level and we have reason to believe that the *sal1* gene may not be the same as multiple aleurone layer (*Mal*) mutation.

Studies of Fe, Zn, and Mn distribution in the mature maize grain revealed that 60 to 80% of the total content of these elements are found in the endosperm, 15 to 35% are found in the scutellum, and 8 to 12% are found in the seed coat [8]. That said, there have been few investigations of micronutrient distribution in maize kernels. Scientists believe that several mineral nutrients in maize are located in the aleurone layer [32], and the possibility of increasing structural and metabolic proteins by selecting for multiple aleurone layers was proposed by the Shewry et al [27].

1.3.2 Improving the Nutritional Quality of Maize through the Aleurone Layer

Sugary (su_1) maize has been reported to contain almost twice as much niacin as the normal starchy kernel(Su_1) [10]. In 1952, the aleurone layer was first investigated as a morphological basis for this increased niacin content [31]: 90% of the kernels assayed from sugary maize with higher niacin had a thicker aleurone layer than the normal starchy kernels.

In the 1970s, a South American race of floury maize ("Coroico" maize), which has an average of 3.7 aleurone layers, was found to have higher total protein content than the normal yellow-dent maize [39]. Nelson and Chang [20] also concluded that in the opaque (*o2*/*o2*/*o2*) endosperm, in the presence of multiple aleurone layers, all amino acids were slightly higher compared to single aleurone layer phenotypes. Nevertheless, they disagree with [39] on the inheritance of the multiple aleurone layer trait. The latter group concluded that the *Mal* is controlled by a single dominant gene while the former group believed that multiple aleurone layer is transmitted as a partial dominant character.

In 1993, it was found that the multiple aleurone layer (*Mal*) gene enhances the effect of the $o2$[10] gene on improving kernel mineral reserves, notably for in Ca, Zn, Cu and Fe [35].

[10] *o2* is a transcriptional factor of bZIP class [28] which controls a group of storage proteins in the maize grains. In 1964, a preliminary result was presented, showing kernels homozygous for opaque-2 (*o2o2*) had 69% more lysine than the normal seeds[17].

1.4 Thesis Approach and Objectives

In the context of this thesis, we studied the effects of multiple aleurone layers' (*Mal*) on maize micronutrient concentrations by means of conventional plant breeding. Related research on *Mal* at Cornell has been going on for over two decades, and focused mainly on increasing iron and zinc concentrations. For instance, kernels with multiple aleurone layer having more Fe, Zn, Mn, Cu and Ca were reported by Welch et al. in 1993 [35].

Ever since Welch et al. published those results [35], there has been ongoing work on breeding for higher mineral (Fe and Zn) density maize, and investigation of Mal on vitamins. Although biofortification through transgenic plants may have the potential to generate greater changes in micronutrient density, biofortification through conventional breeding costs much less, is broadly accepted by societies worldwide, and is expected to benefit human nutrition as well [11].

Our work is based on the assumption that multiple aleurone layer sources are controlled by a single dominant gene, an assumption we share with [20]. Since the *Mal* gene has not yet been cloned, currently we could only distinguish single aleurone layer kernels from multiple aleurone layer kernels by phenotype.

We have assayed our maize kernels segregating for *Mal* across several different inbred backgrounds, in order to see whether our results were consistent with the previous published ones for minerals. Interaction of *o2* and *Mal* is reported on protein content [20], but no published evidence exists regarding the impact of this interaction on mineral concentrations. Vitamins were not assayed in the previous published work on multiple aleurone layer effects on micronutrient density of maize.

[11] `http://www.harvestplus.org/content/tortoise-and-hare-conventional-and-transgenic-approaches-breeding-provitamin-maize`, July, 2009)

The objectives of this thesis are as follows:

1. Determine whether the multiple aleurone layer phenotype maizes have increased mineral and niacin concentration.
2. Determine whether there is any synergistic effect of multiple aleurone layer and opaque-2 (*o2*) on the mineral and niacin concentration.

CHAPTER 2

MINERAL AND NIACIN CONCENTRATION IN MAIZE WITH MULTIPLE VS. SINGLE ALEURONE LAYERS

2.1 Introduction

Micronutrient malnutrition affects roughly 50% of the world population, especially in developing countries and among resource-poor demographics (women and preschool-age children are most vulnerable). It has become a serious socio-economic problem for many of the world's poorest countries and regions, where child mortality rates are high, reproduction is low, people's immune system is impaired, and human capital is decreasing.

In many African and some South-Asian countries, where people are heavily reliant on maize as a dietary staple, micronutrient malnutrition prevails. This is because maize is known for having low levels of bioavailable minerals such as Ca, Fe and Zn [34]. Among people whose main dietary source is maize, pellagra ("rough skin") is common, since maize is not a good source of niacin [30]. We believe that improving the micronutrient quality of maize will be conducive to the reduction of micronutrient malnutrition among the poorest people in the world.

In this chapter, our research was focused on our first hypothesis, asking if the multiple aleurone layer phenotype has an effect on mineral and niacin concentrations. We will report the results of our studies on several mineral nutrients and total niacin concentrations in maize with multiple versus single aleurone layers. We have done nutrient assays in the B8 inbred background and across Mo17 and W64A backgrounds in order to test the hypothesis. We were not able to determine

whether multiple aleurone phenotype kernels were homozygous or heterozygous due to the time limitations inherent to a master's degree project. Thus, the possible dosage effect of multiple aleurone layer alleles could not be studied (the aleurone layer is one of the four triploid tissues in the maize kernel).

2.2 Materials and Methods

2.2.1 Genetic Material

Development of B8 Progenies Segregating for Multiple Aleurone

The source of the multiple aleurone layer (Mal) trait for B8 progeny development was Mo316Mal (obtained from Dr. Larry Darrah at the University of Missouri). Individual plants of Mo316Mal were crossed as female to B8o2 maize plants in 1991. Plants of the F1 were self-pollinated, with selection for Mal and against opaque-2 (o2) in the segregating generations, for three generations.

In summer 2007, seeds with multiple aleurone layers were selected from among the S3 progenies that were uniformly non-opaque (i.e., O2O2 progenies). The number of aleurone layers was determined by hand section in Dr. Ross Welch's laboratory at the USDA's Robert W. Holley Center for Agriculture and Health. The screened seeds were first planted in peat pots in the greenhouse, then hardened off in cold frames and transplanted after about 10 to 14 days to the field in Aurora, NY. Four to five plants per row were self-pollinated to make the S4 generation. In 2007-2008, individual S4 families were backcrossed to B8 in a winter nursery in Florida.

In summer 2008, phenotypic screening was again carried out for number of aleurone layers. Multiple aleurone seeds from individual BC1 ears that were segregating multiple and single aleurone layer kernels were planted in peat pots in the greenhouse. After 10 to 14 days, they were hardened off in cold frames and then transplanted to the field. At least eight plants from each row were then self-pollinated.

The last phenotypic screening was performed in the Histology Unit of the Bailey Hortorium at Cornell University. Kernels with multiple and single aleurone layers from the same BC1S1 ear were identified from rows planted with multiple aleurone seeds. Selected kernels were analyzed for mineral and total niacin concentrations using ICP-AES and UPLC.

Three ears from each of two progeny rows were tested for mineral and total niacin concentrations.

Development of Mo17 and W64A Progenies Segregating for Multiple Aleurone

The source of the Mal trait for Mo17 and W64A progeny development was 5708E Mal*PI5155052 (obtained from the Maize Genetics Cooperation Stock Center). Individual plants of Mal*PI5155052 were crossed as male to the inbreds Mo17 and W64A in summer 2007. The F1 progenies were self-pollinated in the 2007–2008 winter nursery in Florida.

In summer 2008, phenotypic screening (as described above for B8 progenies) was carried out for aleurone layer number determination. Phenotyped kernels from individual S1 ears were planted. Seven to 12 plants per row were self-pollinated.

Selected kernels from three S2 ears from each row were used in the ICP-AES and HPLC assays for mineral and total niacin concentration.

2.2.2 Aleurone Layer Phenotypic Screening and Kernel Drying and Planting Protocol

A more systematic aleurone layer phenotypic screening and planting protocol was developed based on a study of tomato and oat hydration-dehydration in seed germination [7]. The design of a more suitable protocol was necessary in order to allow for the simultaneous planting of large numbers of kernels with known aleurone layer phenotypes. A minimum period of kernel imbibition was required to soften the endosperm and successfully perform hand sections. Once imbibed, kernels either needed to be planted immediately, or preserved in a way that maintained their viability. When a large quantity of seeds must be phenotyped, planting may become prolonged and nonuniform, unless phenotyped kernels can be stored prior to planting. We encountered this problem during the first planting season in summer 2007.

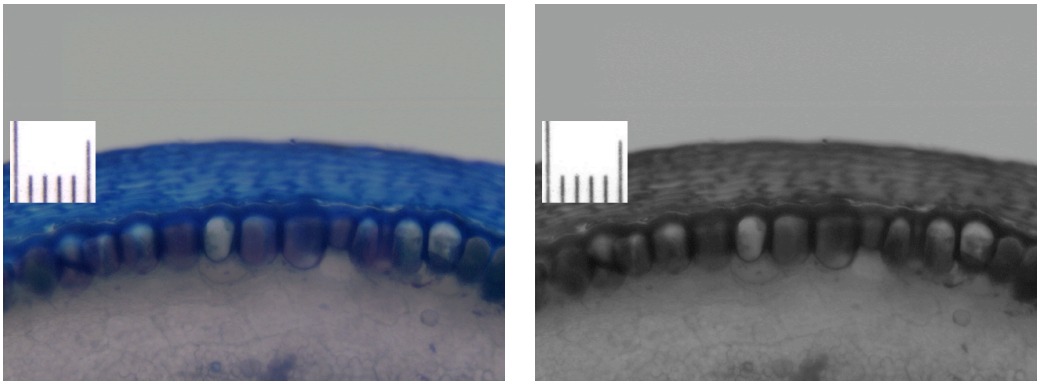
In response, the following protocol was developed and used for the summer 2008 plantings. Target seeds were imbibed in deionized water for 6 to 24 hours, after which hand sections were taken for phenotyping. After screening and selection, the phenotyped seeds were packed by phenotype in envelopes, and dried in the oven at 38°C for 24 to 72 hours. The phenotyped seeds could then be stored for several weeks to allow simultaneous planting of large numbers of seeds in the greenhouse on a single day. This protocol was very successful and resulted in good germination rates as well as excellent transplanting success.

2.2.3 Aleurone Layer Hand Section Methodology

Two methods were used for aleurone layer phenotype screening of the kernels. In the initial phase of the project, screening was carried out in the nutrition lab of the USDA's Robert W. Holley Center for Agriculture and Health at Cornell University. Kernels were imbibed with deionized water overnight. Then the crowns of the individual kernels were cut off using a very sharp razor blade. Finally thin (less than 1 mm) nearly transparent tissue sections were obtained, dyed with Amido Black, and observed under a culture microscope (Olympus CK40-F100, Japan)

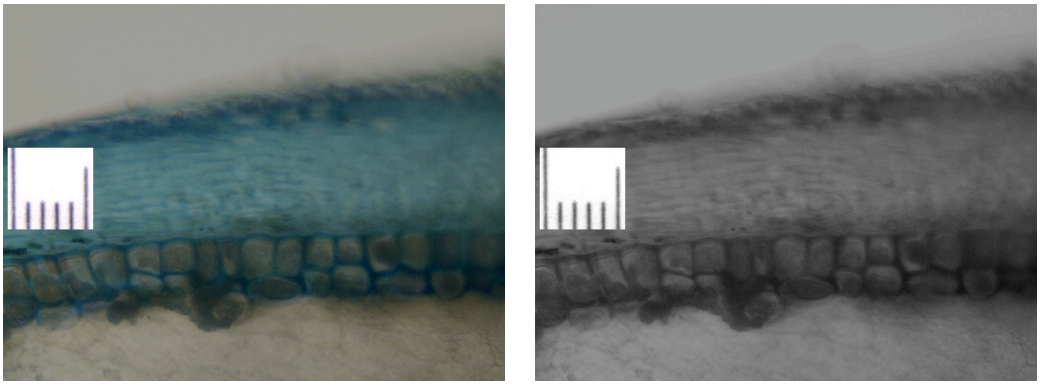
In later phases of the project, aleurone layer phenotyping was carried out in the plant histology unit at Cornell's Bailey Hortorium. Again, the crowns of imbibed kernels were cut using a very sharp razor blade. Then a stick-mounted thin and sharp razor blade was used to take perpendicular, very thin tissue sections off the cut crown under the dissecting microscope. The thin tissue sections were dyed with toluidine blue dye and observed under a compound microscope (Olympus BX60, Japan). We verified that perpendicular crown slice aleurone layer number was the same as aleurone layer number observed in the whole kernel.

Kernels with only one aleurone layer (cf. Figure 2.1) were categorized as single aleurone layer phenotype, and kernels with two (cf. Figure 2.2) or more aleurone layers (cf. Figure 2.3) were categorized as multiple aleurone layer phenotype. Kernels for which the aleurone layer number could not be determined were not used for planting or nutrient analysis.



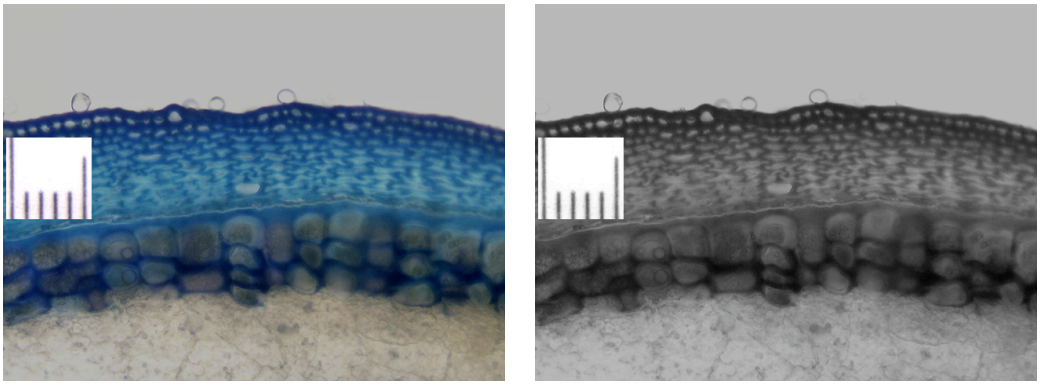
(a) Colored (b) Black and white

Figure 2.1: **Single aleurone layer phenotype from a self-pollinated, segregating ear of Mo17 observed under the microscope, 400×; scale represents 1 mm**



(a) Colored (b) Black and white

Figure 2.2: **Two aleurone layer phenotype from a self-pollinated segregating ear of Mo17 observed under the microscope, 400×; scale represents 1 mm**



(a) Colored (b) Black and white

Figure 2.3: **Three aleurone layer phenotype from a self-pollinated ear of Mo17 observed under the microscope, 400×; scale represents 1 mm**

2.2.4 ICP-AES Mineral Element Assays

Phenotyped and oven dried whole maize kernels were analyzed for grain mineral concentrations using inductively-coupled plasma atomic emission spectroscopy (ICP-AES) (model ICAP 61E trace analyzer, Thermo Electron, Waltham Ma) at the USDA's Robert W. Holley Center for Agriculture and Health Laboratory at Cornell University. Samples were digested with 4.0 mL of concentrated nitric acid at 120°C until dry. Following that, 1.0 mL of a 50/50 mixture of concentrated nitric acid (HNO_3) and perchloric acid ($HCLO_4$) was added and heated at 220°C until dry. After cooling, 0.25 mL of concentrated HCl was added to dissolve the ash. After one hour, the sample was diluted with 10 mL of 5% nitric acid (HNO_3). The ashed sample was mixed and transferred into 15 mL auto sampler tubes and analyzed on an axially viewed ICP trace analyzer emission spectrometer. The transfer optics had been replaced with a short depth of field transfer optics to reduce matrix effects [26].

The output of the ICP-AES was presented in μg/ml (ppm). The final values were multiplied by the volume of dilution (10 mL) and divided by the weight of initial dry maize kernel sample to express results as mg/100g.

Calcium (Ca), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), phosphorus (P), sulfur (S) and zinc (Zn) were analyzed in the maize.

2.2.5 UPLC Niacin Concentration Assay

Sample Preparation

An alkali extraction procedure modified based on a niacin determination in cereal report [14] was utilized. Each kernel was ground separately in a coffee grinder and weighed. Calcium hydroxide and deionized water were added to the individual kernels (around 0.2 to 0.3 g on average) at a proportion of 0.375 g calcium hydroxide accompanied by 10 mL deionized water for every 0.5 g of tissue.

Approximately 100% nicotinic acid (equivalent to the amount of niacin that the individual maize kernels have) was added to the recovery tests (the amount was calculated and estimated from the Strata^{TM}-X-C column optimization described in the following section). The mixture was vortexed thoroughly and heated in an autoclave for 2h at 121°C (approx. 104kPa). The mixture was adjusted to approximately 12 mL using deionized water and allowed to cool down at room temperature. Then the same mixture was brought to 12 mL volume, mixed well and centrifuged at 0°C at 2500 rpm for 15 min. A 10 mL aliquot of the supernatant was taken to adjust to pH=7 using 10% and 1% oxalic acid(Sigma, USA). The pH-adjusted solution was then brought to 35 mL using deionized water. The suspension was centrifuged at 3500 rpm for 20 min to precipitate the calcium oxalate.

A 500 mg C18 Sep-Pak Vac column (Varian, CA, USA) and a 500 mg Strata^{TM}-X-C column (Phenomenex, CA, USA) were connected and conditioned with 10 mL of methanol followed by 10 mL deionized water. A 30 mL aliquot of the supernatant was loaded onto the C18 column. The columns were washed with 10 mL deionized water, then the C18 column was discarded. The Strata^{TM}-X-

C column was washed with 10 mL methanol. Nicotinic acid was eluted from the Strata^{TM}-X-C column into 20 mL scintillation vials (Kimble Glass, INC. NJ, USA) with 10 mL freshly-made prepared 2% solution of concentrated ammonium hydroxide in methanol. The solvent was evaporated to dryness in the ventilation hood (usually 48 to 72 hours was necessary). Finally the residue was dissolved in 3 mL of deionized water. A 300 μL aliquot of each sample solution and several different concentrations of nicotinic acid solutions as standards were prepared for the Ultra Performance Liquid Chromatography (UPLC) assay.

For the samples which had lower than 60% recovery rate, the data was excluded from the analysis.

Strata^{TM}-X-C optimization

Kernels from the F1 of a maize hybrid line were ground using a coffee grinder. For each sample, 0.5 g of ground tissue was mixed with 0.375 g of calcium hydroxide and 10 mL deionized water. Approximately 0.5 g hybrid maize sample was found to have around 10 μg niacin based on previous assays of these same hybrid samples, thus 10 μg niacin solution was added to each sample for the recovery tests.

For the alkali extraction sample preparation, the spike niacin amount was calculated and estimated as the proportion of 10 μg per 0.5 g tissue. The stock solution of nicotinic acid (Acros Organics, NJ, USA) (25 μg/mL) was made in deionized water and stored refrigerated. Working standards were prepared by diluting the stock solution with deionized water.

Among different configurations for the extraction (loading: 10 mL vs 30 mL, and washing and eluting: 5 mL vs 10 mL each) loading with 30 mL washing with

10 mL deionized water and methanol, and eluting with 10 mL eluting solution was found to have the highest recovery rate.

Chromatographic quantification

Chromatographic quantification was performed using a Waters (Milford, Ma, USA) UPLC system equipped with the following Acuity components: Sample Manager, Binary Solvent Manger, PDA Detector, and an HSS T3 1.8 micron 2.1x100 mm reverse phase column.

Ten μL of sample was injected into a 0.7 mL/min eluent stream consisting of 0.1% aqueous trifluroacetic acid (eluent A) and 100% acetonitirile or methanol (eluent B) using the linear gradient profile defined in Table 2.1.

Table 2.1: Linear gradient profile for UPLC niacin concentration assay

Time (min)	Flow (mL/min)	Eluent A (%)	Eluent B (%)
0	0.7	100	0
0.9	0.7	97	3
1.36	0.7	85	15
3.4	0.7	80	20
4	0.7	100	
5.5	0.7	100	

2.2.6 Data Analysis

Data analysis was performed using JMP © 7.0 statistical software. For B8 S4BC1S1 ears, data from two different segregating rows were analyzed together using a model

that included row, phenotype and row × phenotype interaction effects. If the row × phenotype interaction was significant, individual row effects were investigated. For each individual row a statistical model that included ear, phenotype and ear × phenotype effects was used.

For Mo17 and W64A S2 ears, the data was analyzed across inbred backgrounds using a model that included inbred, ear[inbred], phenotype, inbred × phenotype and ear[inbred] × phenotype effects. When inbred × phenotype variation was significant, the effect of the multiple aleurone phenotype in each inbred background was explored using a model that included ear, phenotype and ear × phenotype effects.

2.3 Results and Discussion

2.3.1 Mineral Nutrient Concentrations in Mal vs mal Kernels

In B8 progenies, aleurone phenotype was not a significant source of variation for minerals concentrations, nor was row × aleurone phenotype significant.

Analysis across inbred backgrounds for Mo17 and W64A revealed that copper (Cu) concentration was significantly different between multiple and single aleurone layer kernels (cf. Table 2.2, Figure 2.4). Cu concentration was significantly lower (10%) in the multiple aleurone phenotype kernels than in the single aleurone phenotype kernels.

Table 2.2: **Significance of aleurone layer and its interactions on copper concentration in kernels of Mo17 and W64A.**

Type of Variation	Sources of Variation	P Value
		Cu
Mo17 and W64A	Phenotype	0.02
	Inbred × Phenotype	

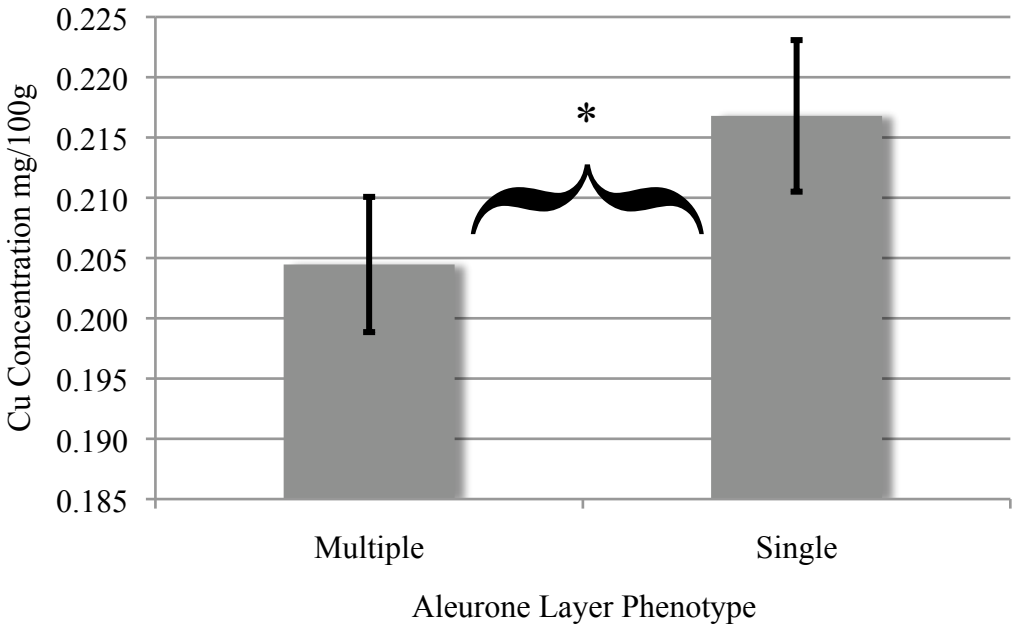


Figure 2.4: **Copper concentration in kernels across Mo17 and W64A inbred backgrounds with different aleurone layer phenotypes (∗P=0.02); vertical bars are standard errors of the mean**

2.3.2 Niacin Concentration in Mal vs mal Kernels

For B8 background, the kernels from two different segregating rows were selected for total niacin concentration assay by UPLC. Unfortunately, because of the low recovery rate (lower than 60%) as noted in Section 2.2.5, we were forced to drop a large portion of the data. Among the reliable data, no significant differences were found in niacin concentration due to aleurone layer phenotype or its interactions.

Mo17 and W64A Progenies

Data obtained for Mo17 and W64A progenies showed that the kernels with multiple aleurone layer phenotype had significantly higher (11.3%) total niacin concentration compared to kernels with a single aleurone layer (cf. Figure 2.5).

Although the inbred × phenotype significance was not detected across the two inbred backgrounds, Test Slice analysis suggested that the significant difference in niacin concentration between multiple aleurone layer and single aleurone layer kernels was mainly contributed by differences in Mo17, where multiple aleurone layer kernels again had higher niacin (23.7%) compared to single aleurone layer kernels (cf. Figure 2.6). The Tukey test and Test Slice results are shown in Tables 2.3 and Table 2.4.

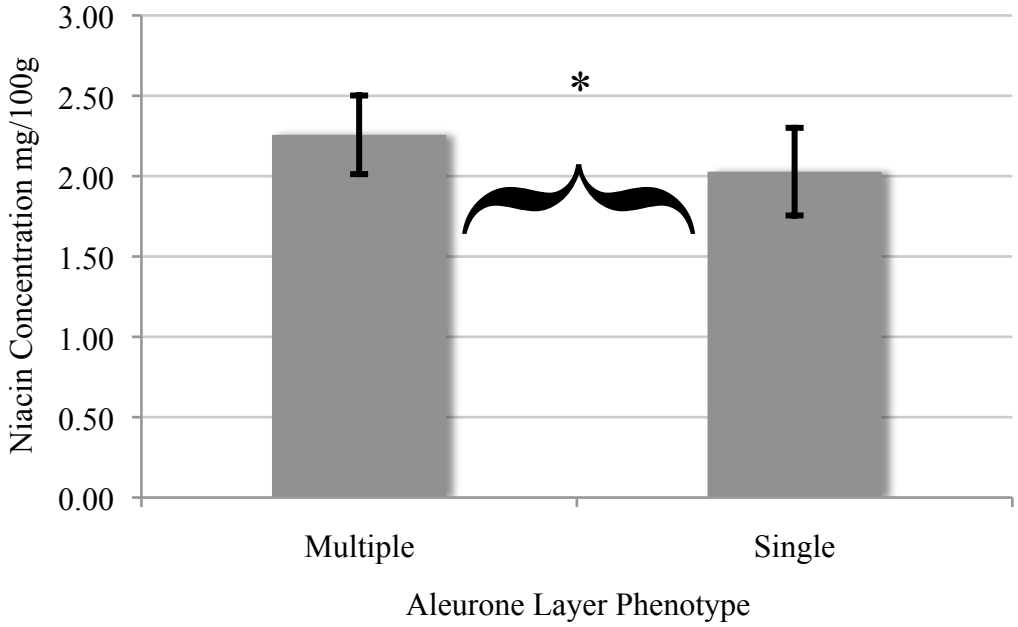


Figure 2.5: **Total niacin concentration in kernels across Mo17 and W64A inbred backgrounds with different aleurone layer phenotypes (∗P=0.0007); vertical bars are standard errors of the mean**

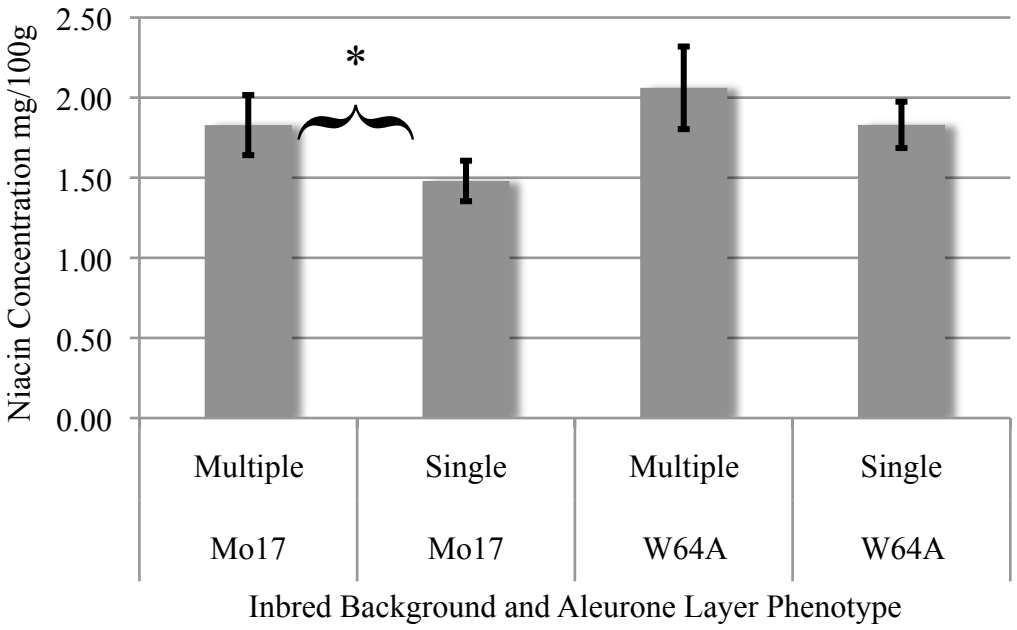


Figure 2.6: **Total niacin concentration in kernels of Mo17 and W64A, respectively, with different aleurone layer phenotypes (∗P=0.001); vertical bars are standard errors of the mean**

Table 2.3: **Tukey test of the inbred by aleurone layer phenotype interaction of total niacin concentration in kernels across Mo17 and W64A.**

LSMeans Differences Tukey HSD					
α=0.050 Q=2.79894					
	LSMean[j]				
LSMean[i]	Mean[i]-Mean[j] Std Err Dif Lower CL Dif Upper CL Dif	Mo17 Mal_	Mo17 malmal	W64A Mal_	W64A malmal
	Mo17, Mal_	0 0 0 0	0.34889 0.09131 0.0933 0.60447	-0.2326 0.10209 -0.5183 0.05318	-0.0014 0.10209 -0.2872 0.28435
	Mo17, malmal	0.3489 0.09131 -0.6045 -0.0933	0 0 0 0	-0.5815 0.10209 -0.8672 -0.2957	-0.3503 0.10209 -0.636 -0.0645
	W64A, Mal_	0.23257 0.10209 -0.0532 0.51832	0.58146 0.10209 0.29571 0.86721	0 0 0 0	0.23117 0.11184 -0.0819 0.54419
	W64A, malmal	0.0014 0.10209 -0.2843 -0.28716	0.35029 0.10209 0.06454 0.63604	-0.2312 0.11184 -0.5441 0.08186	0 0 0 0

Level			**Least Sq Mean**
W64A, Mal_	A		2.06
W64A, malmal	A		1.83
Mo17, Mal_	A		1.83
Mo17, malmal		B	1.48

Levels not connected by same letter are significantly different.

Table 2.4: **Test slice results for inbred by aleurone layer phenotype interaction of total niacin concentration in kernels across Mo17 and W64A.**

Inbred or Aleurone Phenotype (Test Slice)	Comparison	P Value
Mo17	Mal vs. mal	0.001
W64A	Mal vs. mal	0.05
Mal	Mo17 vs. W64A	0.03
mal	Mo17 vs W64A	0.003

2.3.3 Discussion

Mineral Concentration Assay

Across Mo17 and W64A inbred backgrounds, copper was significantly lower (10%) in the multiple aleurone layer kernels. Copper concentration was also reported to be significantly different between multiple and single aleurone layer kernels by Welch and Smith[35]. We could not find nutritional benefits of the multiple aleuron layer phenotype with respect to zinc and iron.

Our genetic materials for nutrient assays were not as nearly isogenic as would have been desired. The multiple aleurone kernels we phenotyped would have included both homozygous and heterozygous genotypes at the *Mal* locus. No clear assessment of the dosage effect, if any, associated with the *Mal* allele has been published. Differences due to any dosage effects could have compromised our results. However, we have provided promising preliminary results that suggests the need for future research. More work is required to verify the trends identified in this thesis. Efforts should be taken to make more nearly isogenic lines and to use bigger sample sizes for the assays. In particular, if true breeding multiple aleurone phenotype kernels could be separated from heterozygous kernels at multiple aleurone locus, the results obtained might be more precise.

There is a potential for enhanced mineral nutrient profile in several inbred backgrounds based on our results. However, further research is required to more precisely document the effects of the multiple aleurone trait in these and a broader range of inbreds.

Total Niacin Concentrations in Mal vs mal Kernels

Our results suggest that across Mo17 and W64A backgrounds niacin concentration was significantly higher (by 11.3%) in the multiple aleurone phenotype kernels. Niacin concentration was also higher (by 23.7%) in the multiple aleurone kernels of Mo17 alone. Vitamin B is confined to certain parts of the maize kernel, in contrast to vitamin B in rice and wheat [11]. If vitamin B accumulation is also related with increased sink size for phloem transport provided by more aleurone layers (as suggested by Welch [35]), the Mal trait is indeed promising for breeding for enhanced niacin in maizes.

On the other hand, that maize is known for not being a good source of niacin is also because niacin is always in a bound form in maize grains, and thus has low bioavailability [30]. When the experiment was designed, acid extraction [38] to assay bioavailable niacin concentration was planned. Unfortunately, the time constraints only allowed us to perform niacin alkali extraction to determine the total niacin concentration.

We suggest that niacin extraction by acid also should be performed and compared to the data obtained by niacin alkali extraction. This would show whether both bioavailable and total niacin increase in association with multiple aleurone layers in a near isogenic background, thus assist to provide more biologically logic results.

The other complication we encountered was the instability of the recovery rate during the sample preparation process. For future experiments, SCX column optimization should be performed regularly.

CHAPTER 3

COMBINED EFFECTS OF THE OPAQUE-2 AND MULTIPLE ALEURONE ALLELES ON MAIZE KERNEL MINERAL AND NIACIN CONCENTRATIONS

3.1 Introduction

Improving micronutrient quality of maize has the potential to reduce micronutrient malnutrition among the poor people in the world.

Biofortification has shown new hope in providing a more sustainable strategy to cope with micronutrient malnutrition among the poor, compared to food fortification and more medically based dietary supplementation. Several research papers on nutrient (mineral, protein, amino acid) concentration enhancement in association with multiple aleurone layers or multiple aleurone layers in the presence of opaque-2 have been published [20, 39, 35].

In this chapter, we aimed to evaluate whether there were synergistic effects on micronutrient concentration between the multiple aleurone layers (*Mal*) and opaque-2 (*o2*) alleles.

3.2 Materials and Methods

3.2.1 Genetic Material

Development of Mo17 and W64A Progenies In Both Normal and Opaque-2 Backgrounds Segregating for Multiple Aleurone

The source of the multiple aleurone layer (Mal) trait was 5708E Mal*PI5155052 (obtained from the Maize Genetics Cooperation Stock Center). Individual plants of Mal*PI5155052 were crossed as male to the inbreds Mo17O2, Mo17o2, W64AO2 and W64Ao2 in summer 2007[1]. The F1 progenies were self-pollinated in the 2007–2008 winter nursery in Florida.

In summer 2008, phenotypic screening (as described in Chapter 2) was carried out for aleurone layer number determination. The opaque phenotype, controlled by the *o2* recessive alleles, was checked on a light desk, with translucent kernels designated as *O2O2* or *O2o2* and opaque ones as *o2o2*. Phenotyped S1 kernels having multiple aleurone layers from individual ears that were segregating for Mal from progenies of Mal*Mo17O2 and Mal*W64AO2 were planted. Opaque S1 kernels that were segregating for multiple aleurone layer were also planted from progenies of Mal*Mo17o2 and Mal*W64Ao2. Seven to 12 plants per row were self-pollinated with the goal of obtaining some ears that were homozygous for Mal, and thus being able to distinguish the effects of Mal in the homozygous versus the heterozygous condition.

[1]In order to distinguish the opaque 2 phenotype better from non opaque kernels in this chapter, non opaque phenotype is designated as O2. Thus we have the inbred Mo17O2 and W64AO2 which is the same as Mo17 and W64A mentioned in Chapter 2

Kernels around the center of the ear were taken to the nutrition lab for micronutrient concentration determination. Due to the limited time available, we only assayed three segregating ears from each of the inbred backgrounds—Mo17O2, Mo17o2, W64AO2 and W64Ao2—to test our hypothesis. Our comparisons were limited to comparing the multiple aleurone phenotype (including both *MalMal* and *Malmal* genotypes) with the single aleurone phenotype (*malmal*) kernels.

Kernel hydration and dehydration and planting protocol as well as the hand section methodology were conducted as described in Chapter2.

3.2.2 Mineral and Vitamin Concentration Assays

We used the same ICP-AES assay for minerals and UPLC assay for niacin as described previously in 2.2.4 and 2.2.5, respectively.

3.2.3 Data Analysis

Data was analyzed by JMP © 7.0 statistical software. Mo17o2 and W64Ao2 were analyzed to see if multiple aleurone layer (Mal) had a significant effect within opaque-2 (o2) inbred backgrounds. Combinations of Mo17o2 and Mo17O2 and of W64Ao2 and W64AO2 were analyzed to investigate the combined effects of *Mal* and *o2*.

In this chapter, aleurone layer phenotype was treated as a phenotypic effect in the analysis and opaque-2 was treated as a component of the inbred background effect. This is because the original sources of the *o2* alleles were versions of Mo17 and W64A that had been converted by others to *o2 o2* type. We have no informa-

tion on how nearly isogenic they are with the wild type Mo17 and W64A sources used.

When tested across the inbred backgrounds carrying the *o2* genotype, we examined aleurone phenotype significance and the intred × aleurone phenotype interaction significance. When the latter was significant, we examined effects of multiple aleurone within individual o2 inbred backgrounds. When both phenotype and the interaction effects were significant, we present the data across the o2 inbred backgrounds, as well as within the individual o2 inbred backgrounds. A similar approach was taken in analyzing the data for Mo17O2 and Mo17o2 combined, and the data for W64AO2 and W64Ao2 combined.

3.3 Results and Discussion

3.3.1 Mineral Nutrient Concentration Assay

Effect of *Mal* Allele in Mo17o2 and W64Ao2

Across Mo17o2 and W64Ao2, magnesium (Mg) concentration was found to be significantly different between multiple and single aleurone phenotype kernels. Mg concentration was 2.0% higher in the multiple aleurone compared to single aleurone layer kernels. (cf. Figure 3.1 Table 3.1)

Potassium (K) concentration showed a significant inbred by aleurone phenotype interaction. Potassium concentration was significantly affected by aleurone phenotype in W64Ao2, but not in the Mo17o2 background (cf. Table 3.1, Figure 3.2). In W64Ao2, K concentration was 6.2 % higher in multiple aleurone kernels

than in their single aleurone layer counterparts.

None of the rest of the minerals were found to have significant variation attributable to Mal phenotype or to the inbred × aleurone phenotype interaction.

Table 3.1: **Significance of aleurone layer phenotype and its interactions on mineral concentrations in Mo17o2 and W64Ao2 kernels**

Type of Analysis	Source of Variation	P Value	
		K	Mg
Mo17o2 and W64Ao2	Phenotype		0.02
	Inbred × Aleurone Phenotype	0.02	
Mo17o2	Phenotype		
	Phenotype × Ear		
W64Ao2	Phenotype	0.03	
	Phenothype × Ear		

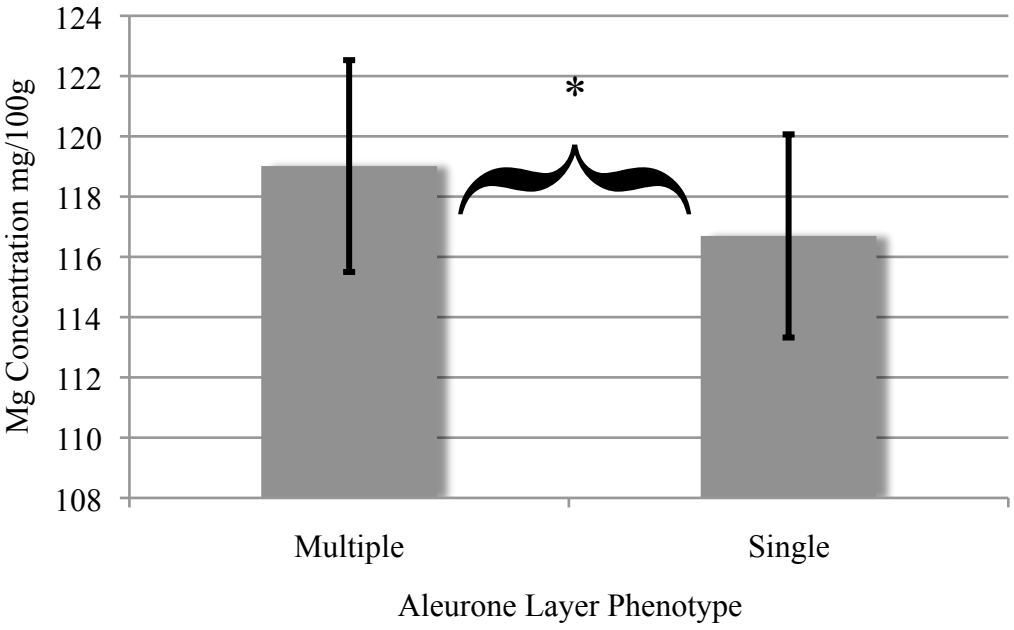


Figure 3.1: **Magnesium concentration in kernels across Mo17o2 and W64Ao2 inbred background with different aleurone layer phenotype (∗P=0.02); vertical bars are standard errors of the mean**

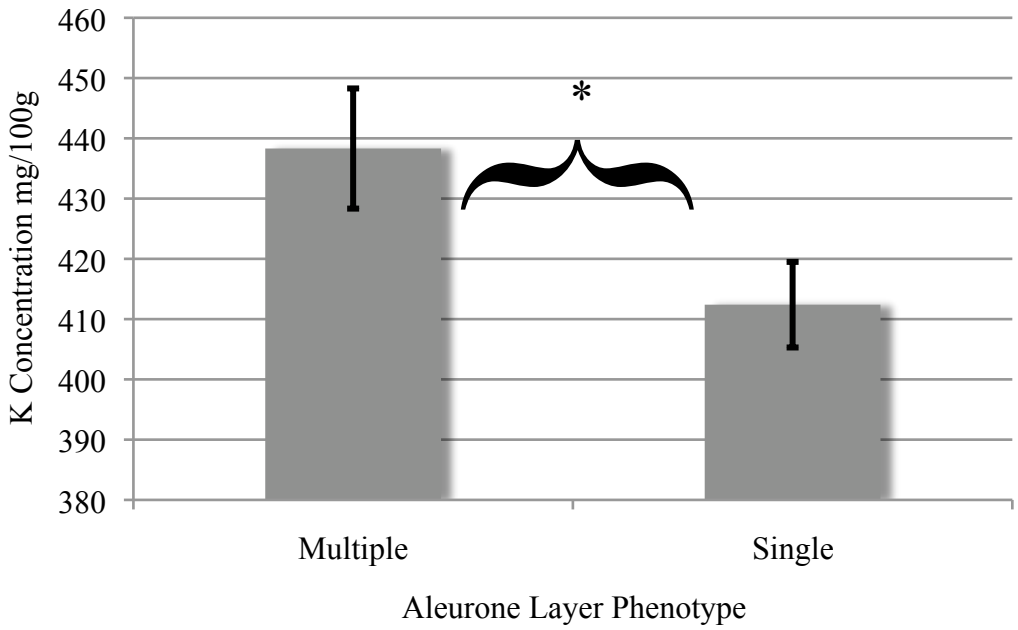


Figure 3.2: **Potassium concentration in kernels of W64Ao2 with different aleurone layer phenotypes (∗P=0.03); vertical bars are standard errors of the mean**

When assayed in the background of W64Ao2 and Mo17o2, the results we obtained were not the same as those we reported in Chapter 2 when the mineral assay was performed across inbred backgrounds of W64AO2 and Mo17O2 (i.e., without opaque-2 in the background). This may be due to interactions between the *Mal* and *o2* alleles, or simply due to genetic background differences between Mo17o2 or W64Ao2 and their wild type counterparts. As noted above, we have no information about how nearly isogenic the source materials that we used actually were.

Mal and *o2* Effects in Mo17O2 and Mo17o2 Inbred Backgrounds

Across Mo17O2 and Mo17o2, none of the minerals showed significant differences attributed to aleurone phenotypes, and none had a significant inbred × aleurone phenotype interaction.

When phenotype was the same, i.e., in kernels with only multiple aleurone phenotype or in kernels with only single aleurone phenotype, the slice test revealed the following trend. For calcium (Ca) and potassium (K), kernels of Mo17o2 had significantly higher mineral concentrations than kernels of Mo17O2 within each aleurone phenotypic class.

For manganese (Mn), phosphorus (P), sulfur (S), and zinc (Zn), within each aleurone phenotype class (Mal or mal), kernels of Mo17o2 had significantly lower mineral concentrations than kernels of Mo17O2.

For iron (Fe), comparing only kernels with multiple aleurone layers, concentration in Mo17o2 is significantly higher than in Mo17O2.

Among the reported mineral concentration alterations by opaque-2 (*o2*), Fe

concentration was numerically increased in the opaque-2 inbred background Mo17o2 with multiple aleurone layers, while the concentration was already increased by opaque-2 in kernels with single aleurone layer. (Mo17o2, Mal > Mo17o2, mal > Mo17O2, mal, data not shown.)

S concentration was numerically further decreased in the opaque-2 inbred background Mo17o2 with multiple aleurone layers, while it was already decreased by opaque-2 in kernels with single aleurone layer. (Mo17o2, Mal < Mo17o2, mal < Mo17o2, mal, data not shown.)

For various minerals, differences in element concentration with or without (*o2*) had been reported before in other inbred lines [35]. However, this was not the main objective of our research project. These results show a potential interaction of opaque-2 (*o2*) and multiple aleurone layers (*Mal*), although not significant.

The numeric trends alone in Mo17 background, especially with certain contradicting results described in the following section (Fe), do not provide strong support for breeding for both *Mal* and *o2* to improve nutritional quality of maize.

Mal and *o2* in W64AO2 and W64Ao2 Inbred Backgrounds

Across W64AO2 and W64Ao2, potassium(K) concentration was significantly lower (7.1%) comparing multiple aleurone versus single aleurone phenotype kernels (cf. Figure 3.3). The slice test (data not shown) revealed that W64Ao2 was contributing the significance (cf. Figure 3.4), which was consistent with the results in the previous section concerning W64Ao2 and Mo17o2. This might explain why, across opaque-2 and non opaque-2 W64A backgrounds, the effect of Mal was opposite from what we observed when mineral concentration was assayed in W64Ao2 alone.

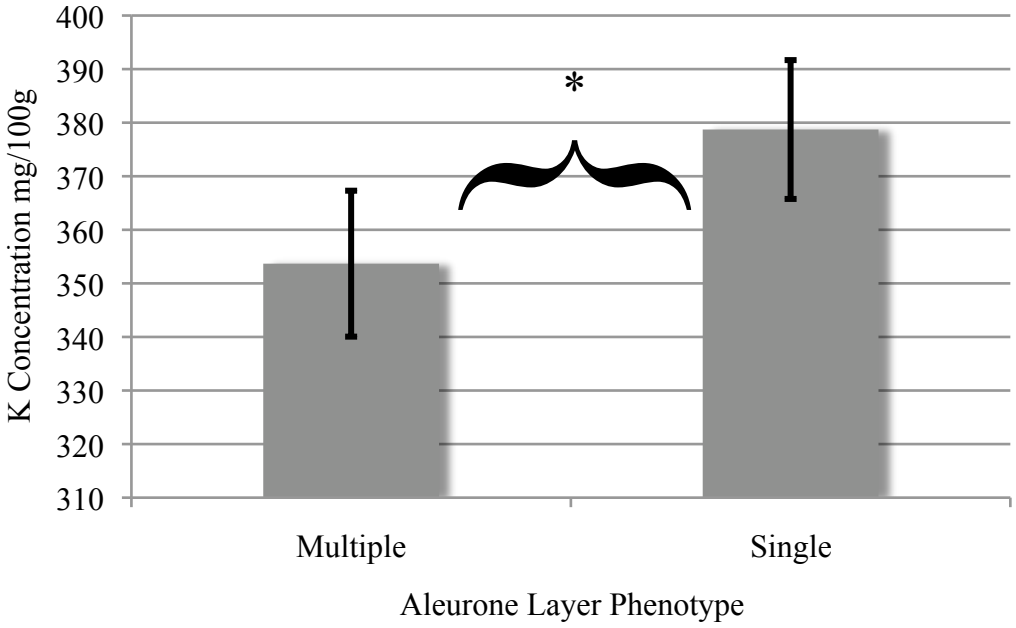


Figure 3.3: **Potassium concentration in kernels across W64Ao2 and W64AO2 inbred background with different aleurone layer phenotypes (∗P=0.01); vertical bars are standard errors of the mean**

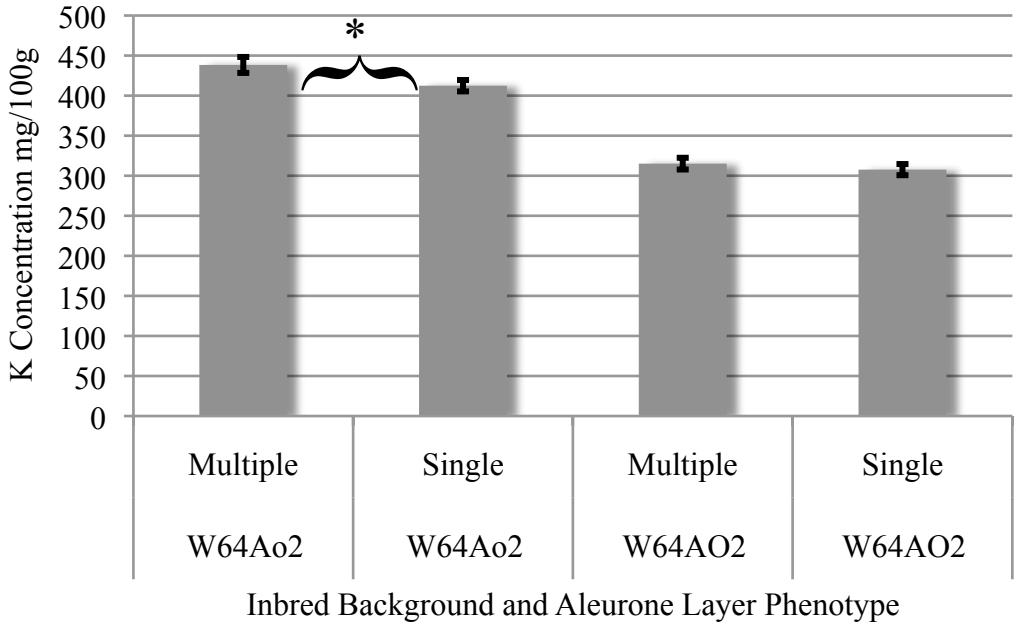


Figure 3.4: **Potassium concentration in kernels of W64Ao2 and W64AO2 respectively with different aleurone layer phenotypes (∗P=0.007); vertical bars are standard errors of the mean**

Manganese (Mn) concentration analysis found the inbred × aleurone phenotype interaction significant. Mn concentration showed significant variation due to aleurone phenotype in the W64AO2 background individually (Figure 3.5).

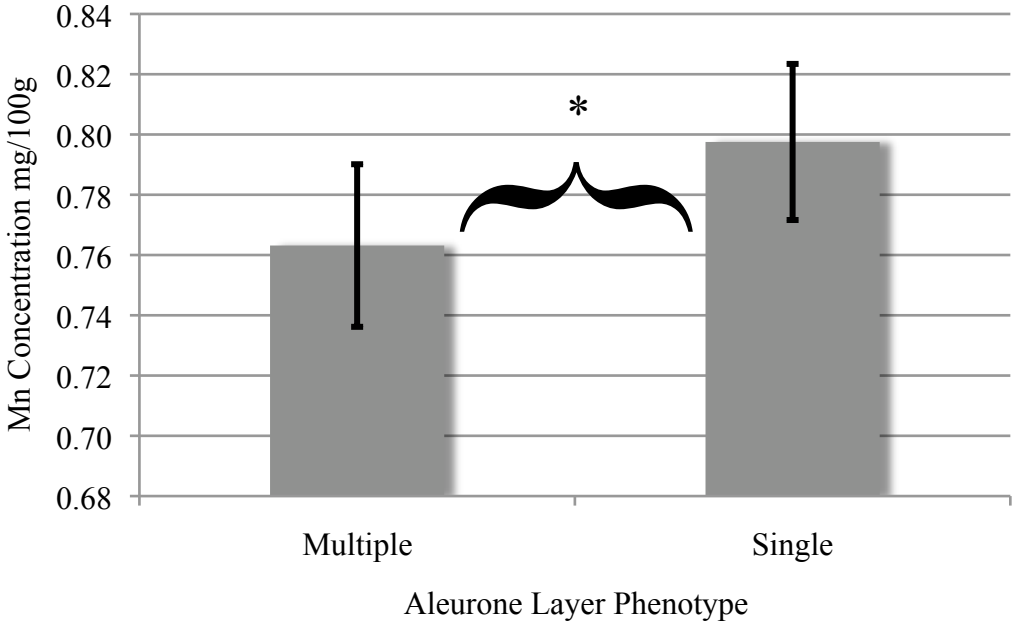


Figure 3.5: **Manganese concentration in kernels of W64AO2 background with different aleurone layer phenotypes (∗P=0.0454); vertical bars are standard errors of the mean**

Test slice of data analysis across W64Ao2 and W64AO2 showed that for copper (Cu), potassium (K), and sulfur (S), within either aleurone phenotypic class, the mineral concentrations were higher in the opaque-2 inbred background than in the non-opaque-2 background.

Iron (Fe), manganese (Mn), and zinc (Zn) concentrations were significantly lower in W64Ao2 than in W64AO2 when aleurone phenotype was held constant. For magnesium (Mg) and phosphorus (P) concentrations in kernels with multiple aleurone phenotype, W64Ao2 was significantly higher than W64AO2.

K concentration *numerically* was further increased in o2 inbred background W64Ao2 with multiple aleurone layers, while the concentration was already increased by the presence of multiple aleurone layers in kernels of non-opaque-2 (W64Ao2, Mal > W64AO2, Mal > W64AO2, mal), and by the presence of o2 in kernels of single aleurone layer phenotype (W64Ao2, Mal > W64AO2, mal > W64AO2, mal).

Fe concentration was also further *numerically* decreased in opaque-2 inbred background W64Ao2 with multiple aleurone layers, while the concentration was already decreased by the presence of multiple aleurone layer in non-opaque-2 kernels (W64Ao2, Mal < W64AO2, Mal < W64AO2, mal, data not shown).

However, Mn concentration was *significantly* further decreased in the opaque-2 inbred background in the presence of multiple aleurone layers when the concentration was already *significantly* decreased by the presence of multiple aleurone layers in the non-opaque-2 W64AO2 inbred background (cf. Figure 3.6).

We conclude that there was a tendency for the *o2* and *Mal* alleles to interact, and for Mn concentration there was significant interaction between the two phenotypes. However, the result showed either non-significant trend in W64Ao2 and W64AO2 backgrounds alone (as compared to Mo17 background) and contradicting (Fe case); or decreased mineral concentration in double mutant. This suggests that the potential for using both *o2* and *Mal* alleles in breeding for enhanced mineral content is limited.

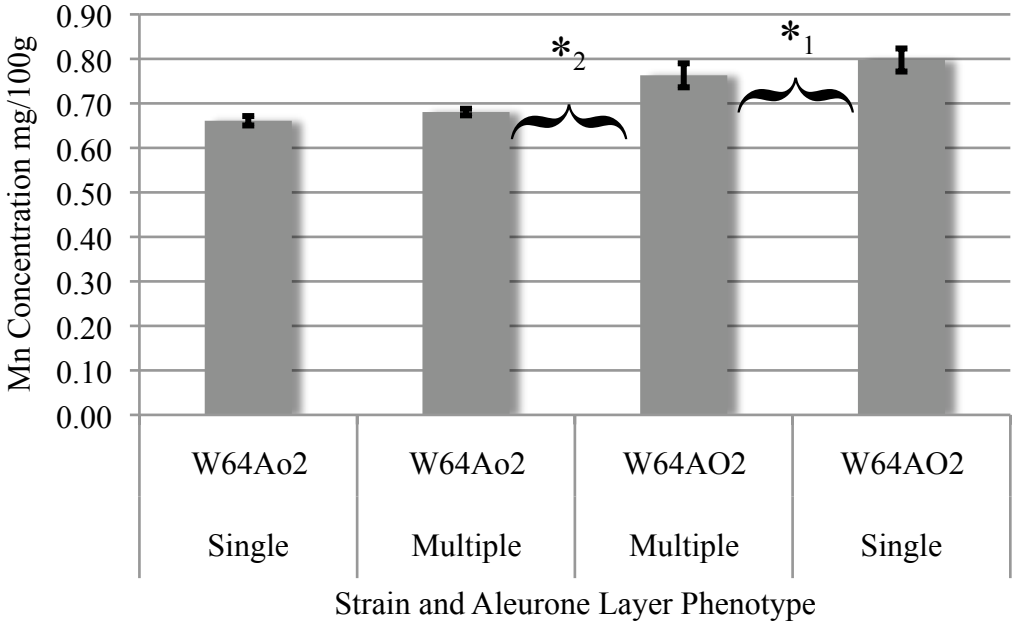


Figure 3.6: **Manganese concentration in W64Ao2 and W64AO2 inbred background with different aleurone layers (significant interaction exists, $*_1$ P1=0.0454, $*_2$ P2=0.007)**

3.3.2 Total Niacin Concentration Assay

The results of niacin concentration assay across Mo17o2 and W64Ao2 were not the same as the niacin assay results from the comparison of Mo17O2 and W64AO2 described in Chapter 2. Aleurone phenotype was not significant across Mo17o2 and W64Ao2 and inbred × aleurone phenotype was not significant either.

Comparing Mo17O2 and Mo17o2, aleurone phenotype was not a significant source of variation, nor was the inbred × aleurone phenotype interaction. That was also true for the comparison of W64AO2 and W64Ao2. Yet, the slice tests from both pairs of analysis indicated that within each aleurone phenotypic class there were significant differences between the opaque-2 background and non-opaque-2 background (cf. Table 3.2, Figure 3.7 and Table 3.3, as well as Figure 3.8).

Table 3.2: **Results from test slice analysis of total niacin concentration interactions for Mo17o2 and Mo17O2 with multiple aleurone (Mal) and single aleurone (mal) layers**

Inbred or Aleurone Phenotype (Test Slice)	Comparison	P Value
Mo17O2	Mal vs. mal	0.06
Mo17o2	Mal vs. mal	0.54
Mal	Mo17o2 vs. Mo17O2	7.60×10^{-6}
mal	Mo17o2 vs Mo17O2	2.69×10^{-7}

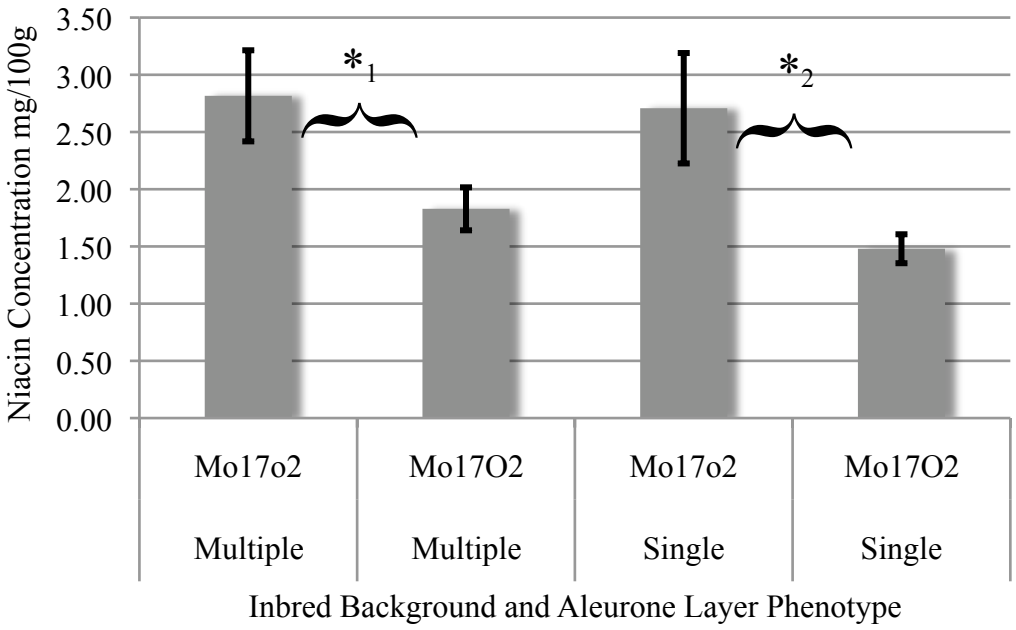


Figure 3.7: **Total niacin concentration in kernels across Mo17o2 and Mo17O2 inbred backgrounds with different aleurone layer phenotypes ($*_1$ P1=7.60×10^{-6}, $*_2$ P2= 2.69×10^{-7}); vertical bars are standard errors of the mean**

Table 3.3: **Results from test slice analysis of total niacin concentrations across W64Ao2 and W64AO2 inbred backgrounds with multiple aleurone (Mal) and single aleurone (mal) layers**

Inbred or Aleurone Phenotype (Test Slice)	Comparison	P Value
W64AO2	Mal vs. mal	0.07
W64Ao2	Mal vs. mal	0.50
Mal	W64Ao2 vs. W64AO2	0.0002
mal	W64Ao2 vs W64AO2	0.002

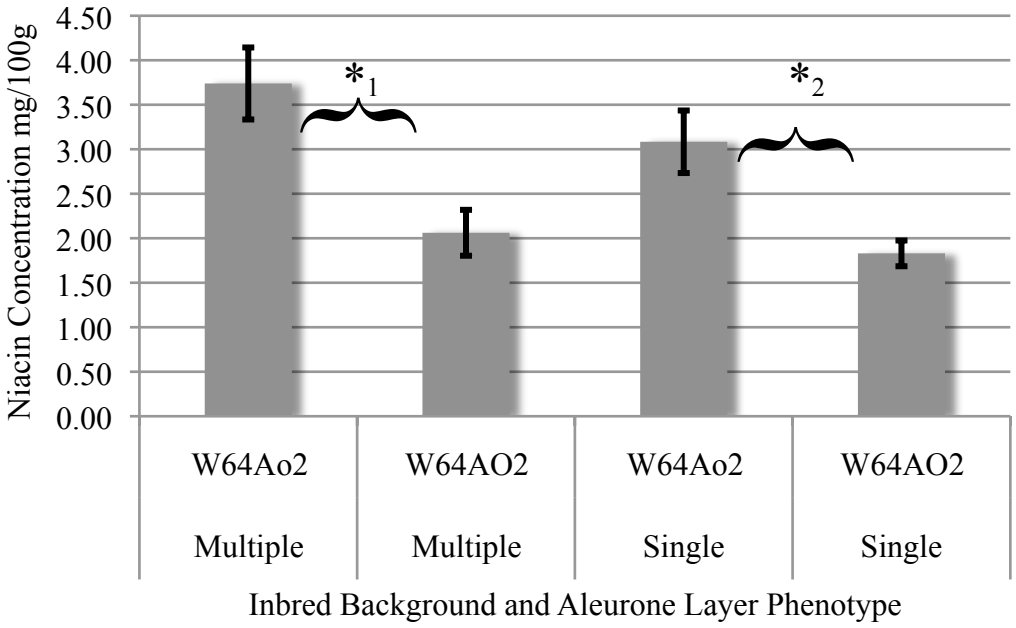


Figure 3.8: **Total niacin concentration in kernels across W64Ao2 and W64AO2 inbred backgrounds with different aleurone layer phenotypes ($*_1$ P1=0.0002, $*_2$ P2=0.002); vertical bars are standard errors of the mean**

Total niacin concentration in kernels of Mo17o2 was significantly higher than in Mo17O2 with multiple aleurone or single aleurone phenotype. Also, niacin concentration in kernels of W64Ao2 was significantly higher than in W64AO2 regardless of the aleurone phenotype. For both Mo17 and W64A, the double mutant added further to total niacin concentration compared to *o2* alone (Mal o2 > mal o2 > mal O2) or compared to *Mal* alone (Mal o2 > Mal O2 > mal O2).

Although the double mutant Mal o2 was not statistically higher in total niacin concentration than the o2 mutant alone, the existence of interaction between *Mal* and *o2* and the numeric superiority of the double mutant, given the heterozygosity remaining in these S2 generation, suggest potential for improving total niacin concentration by incorporating both mutations in some genetic backgrounds.

3.3.3 Discussion

Mineral Concentration Assay

For mineral element concentration assays, the effects of multiple aleurone layers in the Mo17o2 and W64Ao2 were different from the results obtained in the previous chapter, where the assay was performed across Mo17O2 and W64AO2 backgrounds. Since the elements that differed significantly due to aleurone phenotype in these two tests were not the same at all, combined effects of (*Mal*) and (*o2*) could not be estimated based on this result. K and Mn concentrations affected by Mal in the opaque-2 background of W64A additionally supported our first hypothesis in Chapter 2.

While having the same aleurone phenotype, non-opaque-2 kernels showed sig-

nificant difference in mineral concentration versus opaque-2 kernels, our data supports previous results presented by Welch: some maize genotypes with opaque-2 phenotype contain higher or lower mineral concentrations relative to their non-opaque-2 counterparts[35]. Since this is not our major interest, detailed data was not shown.

There was a tendency of synergistic effects due to the combination of opaque-2 (*o2*) and multiple aleurone layer (*Mal*) alleles in Mo17 or W64A (opaque or non-opaque backgrounds). Of W64Ao2 and W64AO2 background, there was significant synergistic effects of *o2* and *Mal* alleles on Mn concentration. Our results on the combined effects of the two phenotypes show a pattern similar to Nelson and Chang's result on amino acid concentration [20]. Our results of mineral numeric trends in different inbred backgrounds and significant result on Mn in W64A (opaque and non-opaque background) however do not provide strong evidence for breeding potential for both *Mal* and *o2* to increase mineral concentration in maize.

The plant material for Mo17o2, Mo17 O2, W64Ao2 and W64AO2 was not pure enough to see the actual difference between different aleurone phenotypes and combined effects of multiple aleurone phenotypes and opaque-2. Further research studying more nearly isogenic lines is necessary to verify our data.

The multiple aleurone layer phenotype was the focus of comparison between Mo17o2 and Mo17O2 and between W64Ao2 and W64AO2. Few minerals showed significant variation attributable to Mal trait, except for K in W64Ao2 and Mn in W64AO2. The small sample size disadvantage could not be excluded.

Total Niacin Concentration Assay

Total niacin concentration was not significantly affected by *Mal* when tested across Mo17o2 and W64Ao2. This is in contrast to what we observed in the non-opaque background reported in Chapter 2. This suggests the existence of interaction between *Mal* and *o2* affecting total niacin concentration. These results are complicated by possible genetic differences between opaque and non-opaque inbred sources. For both Mo17 and W64A, there was a numeric tendency for the double mutant to have the highest total niacin concentration. This suggests that incorporating both *Mal* and *o2* into a homozygous inbred background has the potential to increase total niacin concentration for that inbred.

CHAPTER 4

CONCLUSION

4.1 Summary

4.1.1 Effect of The Multiple Aleurone Layer (*Mal*) on Mineral and Total Niacin Concentrations of Maize Kernels

The first hypothesis we tested was the presence of multiple aleurone layers has an impact on mineral and niacin concentrations. For nutritionally important mineral elements, we found no significant effects due to the presence of *Mal* in the B8 background. Across Mo17 and W64A, copper concentration was lower in multiple aleurone layer compared to single aleurone layer kernels. Data was insufficient to assess *Mal* effects on niacin in the B8 background, however, niacin concentration was about 11.3% higher in *Mal* kernels when analyzed across Mo17 and W64A backgrounds. Progenies from the Mo17 inbred background were primarily responsible for the average increase in niacin concentration. In this particular inbred, niacin concentration was 23.7% higher in multiple versus single aleurone layer kernels.

4.1.2 Combined Effects of Multiple Aleurone Layers (*Mal*) and Opaque-2 (*o2*) on Mineral and Total Niacin Concentrations of Maize Kernels

Among opaque-2 progenies across Mo17o2 and W64Ao2 inbred backgrounds, magnesium concentration was significantly higher in kernels with multiple aleurone versus single aleurone phenotype. Potassium concentration was significantly higher for multiple as compared to single aleurone kernels only in the W64Ao2 inbred background. The effects of *Mal* on mineral concentrations differed between this analysis, which was done in the *o2* mutant endosperm, and the analysis reported in Chapter 2, which was done in a normal endosperm background. Since these differences related to minerals that were significantly altered by *Mal* in one endosperm type but not the other, this does suggest a possible interaction between these two alleles (*Mal* and o2).

The presence of opaque-2 in otherwise nearly isogenic lines significantly altered several mineral concentrations in both Mo17o2 vs. Mo17O2 and W64Ao2 vs. W64AO2. For several minerals (iron, potassium, and sulfur), there was a numeric tendency suggesting a synergistic effect of opaque-2 and multiple aleurone layers in Mo17 and W64A backgrounds with or without opaque-2, respecitvely; however, only manganese concentration in the W64A background showed a significant interaction (W64Ao2, Mal < W64AO2, Mal < W64AO2, mal).

Total niacin concentration was not found to be significantly different in multiple aleurone versus single aleurone layer kernels across Mo17o2 and W64Ao2. However, interaction was apparent for niacin concentration in both Mo17 and W64A backgrounds. In the case of Mo17, the mean niacin concentration was numerically

highest in the double mutant, slightly less in Mo17o2 mal, notably less in Mo17O2 Mal, and again slightly lower in the wild type. All differences were at or near significance (highest P value was 0.06) except the difference between the multiple and single aleurone phenotypes in the opaque-2 endosperm. For W64A, the numerical order of the genotype means was identical to that noted above for Mo17, again with all differences at or near significance (highest P value was 0.07) except the difference between multiple and single aleurone phenotype in the opaque-2 endosperm.

In conclusion, since none the minerals showed significant difference in kernels of multiple aleurone versus single aleurone layer, in both opaque-2 as well as non-opaque-2 background; yet numeric trends only appeared in one of the backgrounds (Mo17 and W64A) or simply contradicting; the significant trend we obtained for Mn in W64A background decreased, the potential of breeding for both *o2* and *Mal* for increasing mineral concentration in maize is open to question. However the numeric trend described earlier for total niacin concentration, which reflects higher concentration in the double mutant as compared to either of the single mutant genotypes, and where difference was at or near the significance threshold, suggests that at least for total niacin this may be a promising breeding avenue to pursue.

4.2 Reflection

During the three year project carried out in the field and laboratory, there have been many things that we have learned that may provide additional explanations for our results and may be of benefit to future research as well.

Back in the field in the summer of 2007, we did not realize the importance and possibility of studying the homozygosity of the PI5155052*Mal source obtained from the Maize Cooperation Stock Center. Thus, the plants we self-pollinated in PI5155052*Mal were not the same as the ones used as males to be crossed with our inbreds. Had we used the same plants, we could have answered whether Mal was homozygous or heterozygous in our source plants.

Instead we attempted to clarify the homozygosity or heterozyosity of Mal in our progenies when the plants were harvested in fall of 2007. We performed a phenotypic screening of the self-pollinated progenies of PI5155052*Mal. Given 20 kernels of one ear, if none of the kernels exhibit the single aleurone layer phenotype, we classified the ear as homozygous. We had been told that the Mal source from the stock center was characterized by phenotype evaluation only. Our investigation results further suggest that the Mal source PI5155052*Mal was from a heterogeneous population. Not having controlled the PI5155052*Mal source in the multiple aleurone introgression into the single aleurone layer phenotype kernels made the screening for the 2008 planting difficult.

When the plants were harvested from 2007–2008 nursery in Florida, we needed to separate the opaque-2 kernels from the non-opaque kernels. This was necessary for planting true breeding opaque-2 kernels during the summer of 2008, and for the combined effects tests. To our surprise, the ears segregating for opaque-2 did not seem to segregate in the same pattern for multiple aleurone layer as those ears that were not segregating for the opaque-2 phenotype.

Oddly enough, the ears were also not segregating at a 3:1 ratio of normal kernels versus opaque kernels. It appeared that in some inbred backgrounds, only in ears that were not segregating at a 3:1 ratio of normal versus opaque-2 kernels could we

find segregation for multiple aleurone layer and single aleurone layer kernels. We believe it is critical to study the inheritance of PI5155052*Mal and its potential interaction with opaque-2 in the future. The literature review we conducted also suggests the existence of opaque-2 modifiers [23, 40], which may have played a role in the odd segregation ratios that we observed.

We also came across research suggesting that aleurone cells secrete hydrolases to mobilize nutrients in the endosperm after 24 hours of imbibition [25]. So when preparing samples for the mineral and niacin concentration assays in the oven, we made every effort to control imbibition and dehydration. Nevertheless, it was not known how kernel micronutrient composition had changed *within* the first 24 hours.

What is more, recovery rate of niacin extraction varied strongly from one batch of kernels to the next. We only kept the data from batches with a recovery rate above 60%. In the future, regular optimization on the Strata$^{\text{TM}}$-X-C column is recommended.

Finally, we would like to note that this turned out to be a very labor-intensive project. This can be attributed to the fact that we are still at the beginning of understanding the effects of multiple aleurone layer on micronutrient concentration. Tools for automating the process of phenotyping will be of great benefit to future research if such tools can be developed

4.3 Future Research

We already emphasized the importance of having plant material of nearly isogenic lines in Chapters 2 and 3. To be precise, when the plants used are true-breeding, they can be ground together for the mineral and niacin assays. In other words, it will not be necessary to phenotype every single maize kernel for multiple aleurone and single aleurone layers. This would also allow a much larger sample size, increasing the confidence in one's results. Thus for the future, researchers should focus on making nearly isogenic plants that are homozygous for the four possible genotype combinations at the *Mal* and *o2* loci.

We also suggest studying the amino acid concentration due to aleurone phenotypes and combined effects of *Mal* and o2 of the plant material used in this thesis. Of particular interest here is whether the results of such a study would support the the conclusions in previous research [20, 39], i.e., whether total protein and amino acid concentrations are altered in the presence of multiple aleurone layers.

Furthermore, opaque-2 and multiple aleurone layer interaction, as well as opaque-2 modifier studies could be carried out with the plant material we harvested from the 2007–2008 winter nursery.

We had originally obtained two more Mal sources from the Maize Genetics Cooperation Stock Center. The first one is Mal*Galinat with a sugary background. The second one is Mal*Nelson, which segregates for purple and yellow kernel color. These two lines are more difficult to screen for multiple aleurone, because of their wrinkly coat and purple color, respectively.

Mineral and micronutrient distribution within mature maize kernels has been a research focus for decades. We suggest that studies on alterations of micronutrient

concentrations for multiple aleurone layers be combined with studies on micronutrient distribution. The study we initiatied on embryo size effects on micronutrient concentration in maize kernels could contribute important insights into this topic too.

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